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Sequences (MIPS) databases were reconciled. Ty elements and dubious open reading frames (ORFs) were excluded. The data set (5,790 proteins) and search results can be viewed at the URL <http://acer.gen.tcd.ie/~khwolfe/yeast>. Repetitive regions within proteins were masked using the SEG filter in BLAST. **Statistical analysis.** Chi-square tests (data not shown) indicate that duplicated genes in yeast are distributed in a highly non-random manner with regard to both the order in which homologous genes occur on pairs of chromosomes and the transcriptional orientations of those genes. A simultaneous origin of duplicate regions, as opposed to 55 independent duplications, is supported by a chi-square test on block orientations and by the lack of triplicated regions. The Poisson expectation if blocks were duplicated sequentially is for approximately 40 duplicated blocks, and 7 blocks that are replicated more than once (mainly triplicated). There is only one possible candidate for a triplicated region: the genes *YDR474C*, *YDR492W* and *GNP1* on chromosome IV and *YOR019W*, *YOL002C* and *SCM2* on chromosome XV meet our criteria for a duplicated chromosomal region; this is not shown in Fig. 2 because this area of chromosome IV overlaps with blocks 18 and 9, which have a higher density of homologues than the proposed chromosome IV/XV block. The three-gene match between chromosomes IV and XV is probably spurious, but even if this is counted as a triplication the departure from Poisson expectations is significant ( $P = 0.001$ ).

**Phylogenetic analysis.** Protein sequences were aligned using default settings in ClustalW with manual editing to remove regions whose alignment was not clear. Branch lengths were estimated with correction for multiple hits<sup>25</sup>. The mean age of duplication was estimated as  $(B/(A+B) + C/(A+C))/2$ , where A, B and C correspond to the lengths of branches A, B and C shown in Fig. 3. Confidence intervals were estimated by bootstrap analyses for genes where there were >10 inferred substitutions on branch A. One gene pair, *ORC1/SIR3*, was omitted because one of the yeast genes appeared more similar to its human homologue than to its duplicate.

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Correspondence and requests for materials should be addressed to K.H.W. (e-mail: [khwolfe@tcd.ie](mailto:khwolfe@tcd.ie)). Additional details of the dataset and results are available on the World-Wide Web at URL <http://acer.gen.tcd.ie/~khwolfe/yeast>.

## A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells

Gosse J. Adema\*, Franca Hartgers\*, Riet Verstraten\*, Edwin de Vries\*, Gill Marland\*, Satish Menon†, Jessica Foster†, Yuming Xu†, Pete Nooyen†, Terrill McClanahan†, Kevin B. Bacon† & Carl G. Figdor\*

\* Departments of Tumour Immunology and † Pathology, University Hospital Nijmegen St Radboud, Philips van Leydenlaan 25, 6525 EX Nijmegen, The Netherlands

† Departments of Immunology and Molecular Biology, DNAX Research Institute, Palo Alto, California 94304, USA

Dendritic cells form a system of highly efficient antigen-presenting cells. After capturing antigen in the periphery, they migrate to lymphoid organs where they present the antigen to T cells<sup>1,2</sup>. Their seemingly unique ability to interact with and sensitize naive T cells gives dendritic cells a central role in the initiation of immune responses and allows them to be used in therapeutic strategies against cancer, viral infection and other diseases. How they interact preferentially with naive rather than activated T lymphocytes is still poorly understood. Chemokines direct the transport of white blood cells in immune surveillance<sup>3,4</sup>. Here we report the identification and characterization of a C-C chemokine (DC-CK1) that is specifically expressed by human dendritic cells at high levels. Tissue distribution analysis demonstrates that dendritic cells present in germinal centres and T-cell areas of secondary lymphoid organs express this chemokine. We show that DC-CK1, in contrast to RANTES, MIP-1 $\alpha$  and interleukin-8, preferentially attracts naive T cells (CD45RA<sup>+</sup>). The specific expression of DC-CK1 by dendritic cells at the site of initiation of an immune response, combined with its chemotactic activity for naive T cells, suggests that DC-CK1 has an important rule in the induction of immune responses.

Dendritic cells are key regulators in immune responses, capable of priming naive T cells. Their potent antigen-presenting capacity can be explained in part by their unique life cycle and their high expression of major histocompatibility complex (MHC) class I and II molecules as well as co-stimulatory molecules<sup>1</sup>. Detailed molecular analysis of dendritic cell function has been hampered, however, by the low numbers of dendritic cells present in blood mononuclear cells. The mechanism by which dendritic cells interact with or activate resting naive T cells to initiate an immune response is not fully understood. One possibility is that secreted cytokines or chemokines preferentially attract or activate naive rather than activated T cells. We generated sufficient numbers of dendritic cells *in vitro*<sup>5,6</sup> to prepare a panel of dendritic-cell cDNA libraries, which allowed us to analyse dendritic cells at the molecular level.



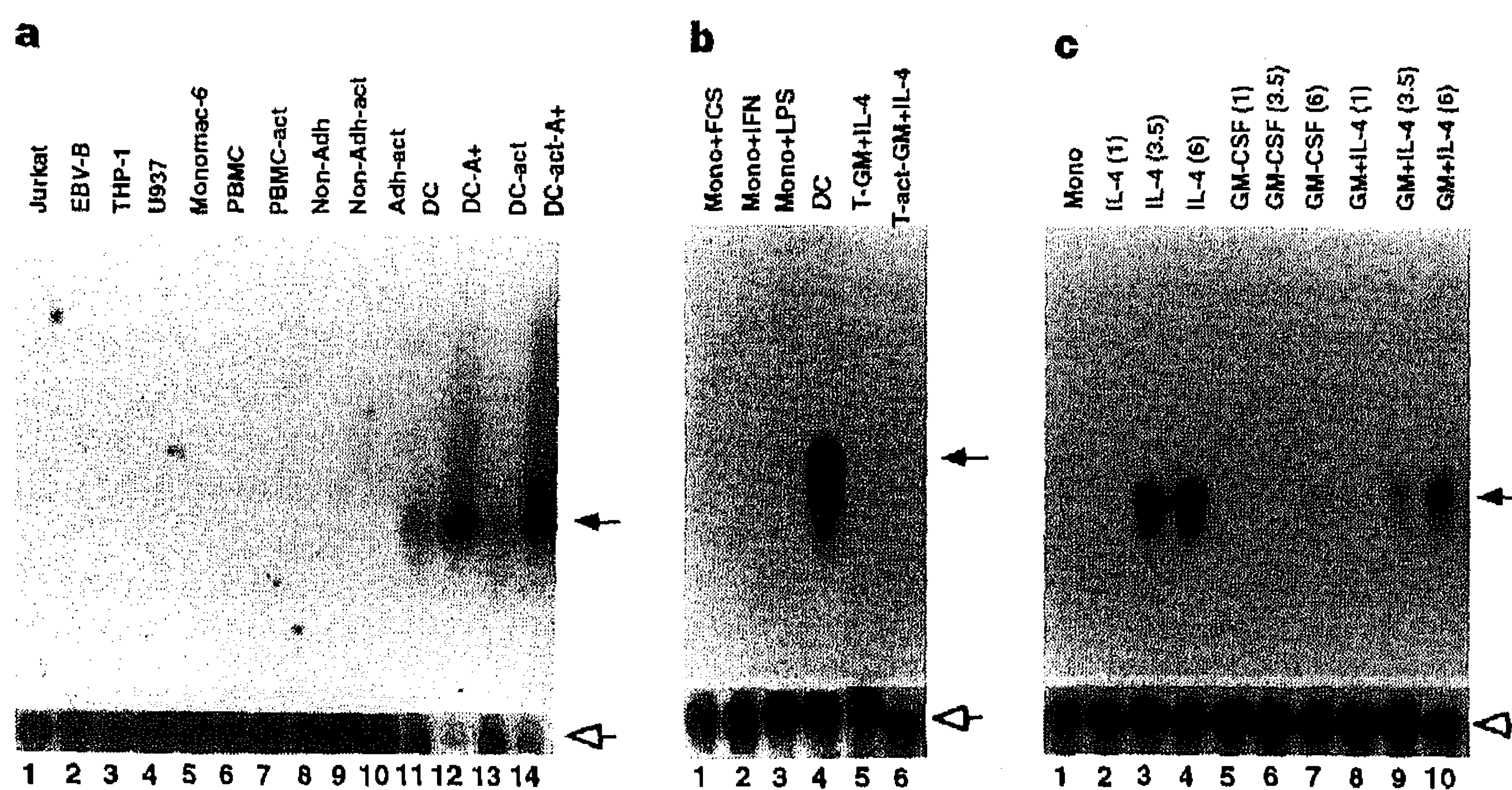
While searching at the genetic level for molecules expressed by dendritic cells, we identified a cDNA clone encoding a C-C chemokine that is abundantly expressed. The deduced sequence predicts a protein of 89 amino acids, of which the amino-terminal 20 amino acids contain all the characteristics of a signal peptide (Fig. 1a). The mature protein comprises 69 amino acids and is 63%, 38% and 33% identical to the chemokines MIP-1 $\alpha$ , RANTES and MCP-2, respectively (Fig. 1b). The idea that this chemokine is expressed abundantly by dendritic cells is supported by the finding that of 350 dendritic cell cDNAs analysed, six encode this C-C chemokine. We termed it DC-CK1.

An RNA species of 1.1 kb was readily detected by northern blot analysis in both total and poly(A)<sup>+</sup> RNA isolated from dendritic cells (Fig. 2a). No DC-CK1 expression could be detected in T cell-, B cell- or three different monocyte cell lines. Moreover, freshly isolated resting or phytohaemagglutinin (PHA)/interleukin (IL)-2

activated peripheral blood mononuclear cells (PBMCs), the resting or PHA/IL-2 activated non-adherent PBMC fraction (T, B and NK cells), and the adherent PBMC fraction (monocytes) activated with lipopolysaccharide (LPS) all failed to express this chemokine. Two endothelial cell lines, representatives of a non-leukocyte cell type known to be capable of expressing a variety of chemokines<sup>3,4</sup>, did not express the chemokine either (data not shown). Because dendritic cells abundantly expressing DC-CK1 were derived from purified monocytes cultured in the presence of GM-CSF and IL-4 (refs 5–7), we tested the ability of different stimuli to induce its expression in monocytes. Fetal calf serum (FCS), interferon (IFN)- $\gamma$  and LPS were all unable to induce DC-CK1 mRNA (Fig. 2b). To demonstrate further the specific induction of this chemokine by GM-CSF plus IL-4 in monocytes, we cultured purified T cells in the presence of GM-CSF plus IL-4, with or without PHA/IL-2. No DC-CK1 mRNA could be detected in T cells under either of these



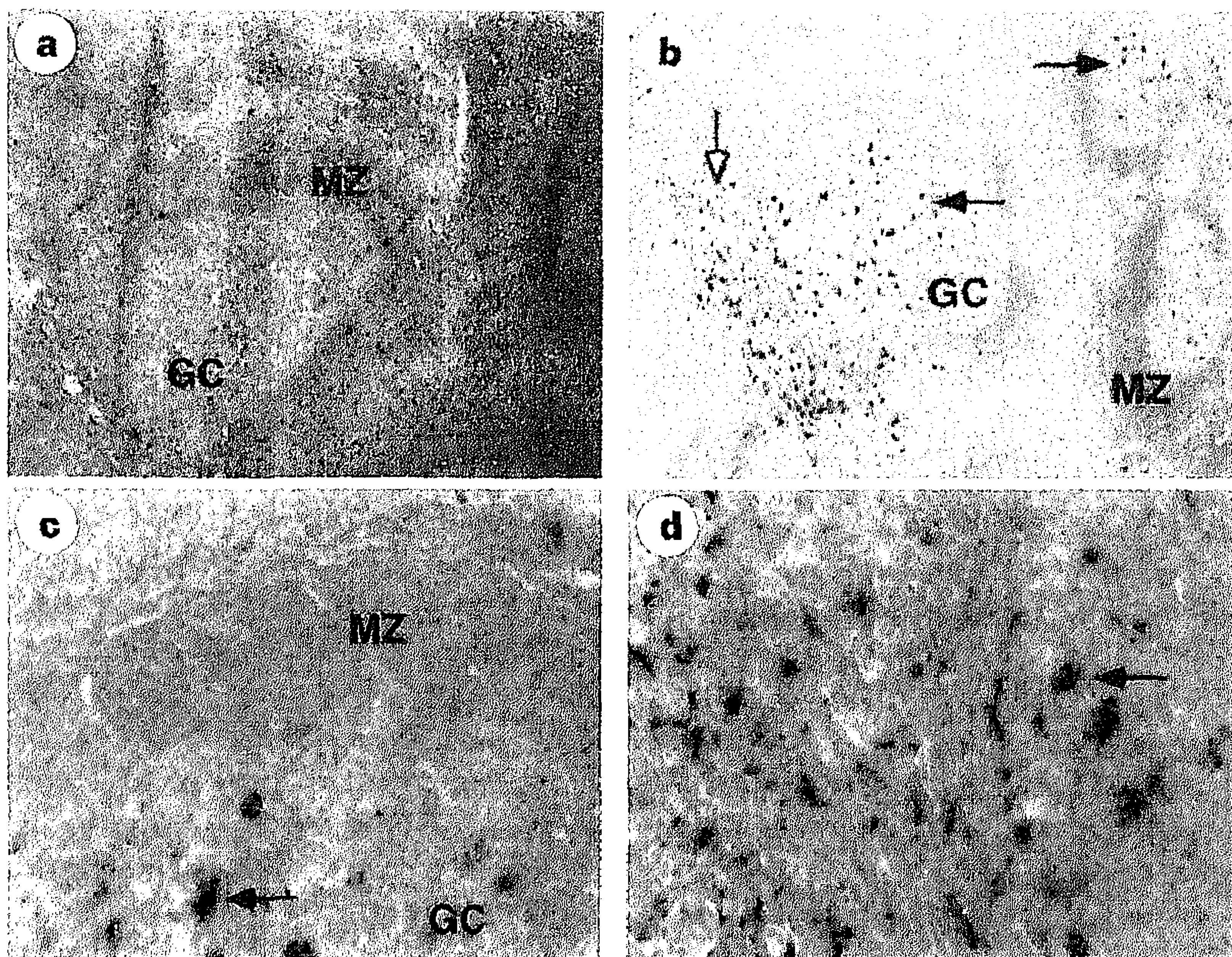
**Figure 1 a**, Nucleotide and amino-acid sequence of DC-CK1. Characteristic cysteines and the experimentally determined signal peptide are in italics. The non-consensus poly(A) site is underlined. **b**, Amino-acid comparison of mature DC-CK1 with the C-C chemokines MIP-1 $\alpha$ , MCP-2 and RANTES. The characteristic cysteines are shown in bold. Identical amino acids (dashes) and gaps (asterisks) introduced for optimal alignment are indicated.



**Figure 2** DC-CK1 mRNA is specifically expressed by dendritic cells. **a**, Northern blot analysis of DC-CK1 expression (filled arrow) in T (Jurkat), B (EBV-B) and monocyte \*THP-1, U937, MonoMac-6) cell lines, freshly isolated resting or activated (act) PBMC subsets and GM-CSF plus IL-4-generated dendritic cells before (DC and D-A<sup>+</sup> for polyA<sup>+</sup> RNA) and after activation with TNF- $\alpha$  plus IL-1 $\alpha$  (DC-act and DC-act-A<sup>+</sup> for polyA<sup>+</sup> RNA) as indicated above each lane. **b**, Northern analysis of DC-CK1 expression in monocyte-derived dendritic cells and freshly isolated monocytes (mono) stimulated as indicated and T cells cultured in GM-

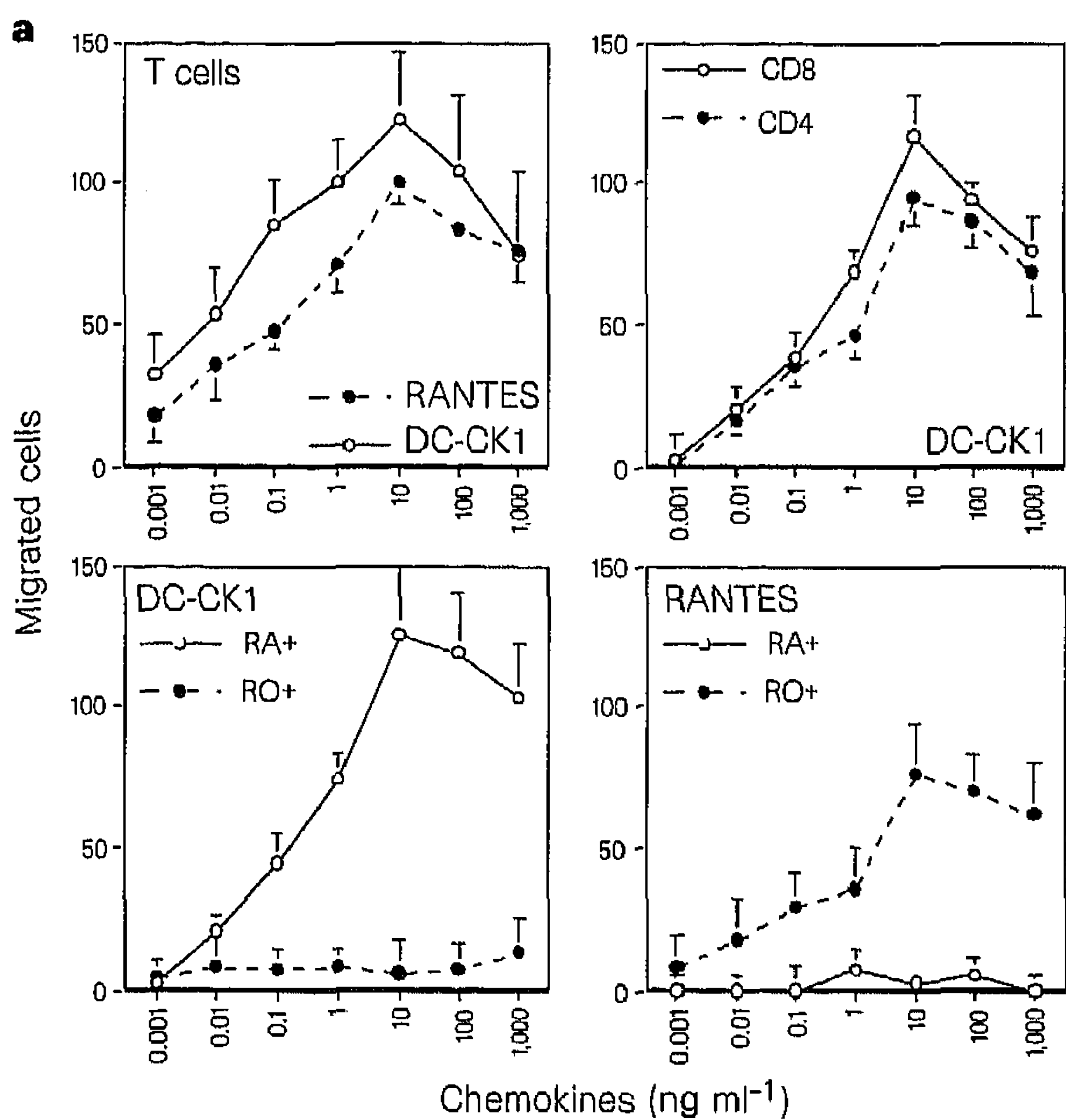
CSF plus IL-4 with or without activation with PHA/IL-2. **c**, Time course of DC-CK1 mRNA expression in freshly isolated monocytes cultured in the presence of IL-4, GM-CSF or the combination for the time (days) indicated in brackets. Northern blots were hybridized to a DC-CK1-specific and a control 28S ribosomal (open arrow) probe. Analogous results were obtained when cDNA libraries prepared from most of the same cell types were examined on Southern blots after digestion to release their inserts (not shown).



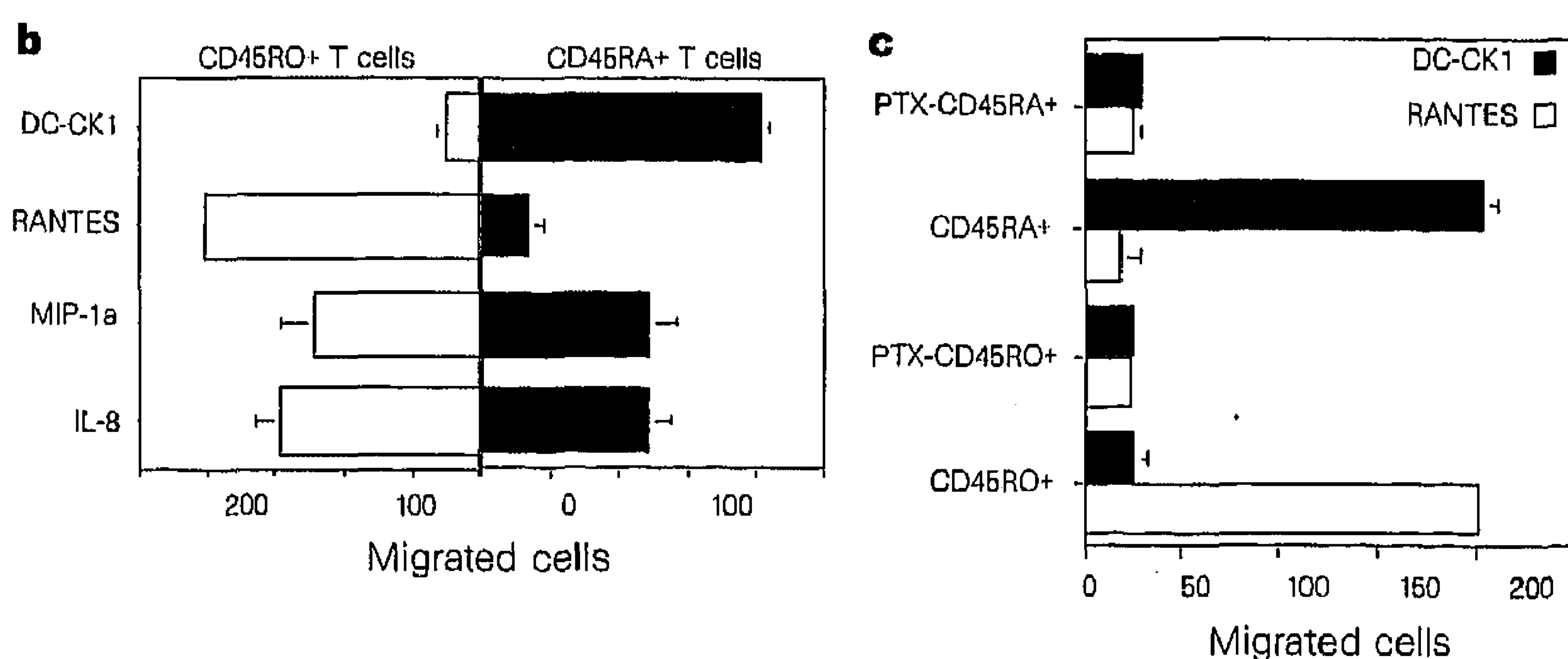


**Figure 3** Detection of DC-CK1 in germinal centres and T-cell areas of a tonsil by *in situ* hybridization. Tonsil sections were stained with a sense (a) or antisense (b-d) DIG-labelled DC-CK1 RNA probe. Magnifications are  $\times 40$  (a, b) and  $\times 200$  for c (germal centre) and d (T-cell area). The filled arrow indicates staining in the

germal centres (GC) and the open arrow in a T-cell area. No staining was observed in the mantle zone (MZ). An incubation time of 2-3 h with NBT/BCIP was sufficient to detect DC-CK1 mRNA, which is consistent with the abundance of DC-CK1 in the cDNA libraries.



**Figure 4** DC-CK1 is a potent chemoattractant for the CD45RA<sup>+</sup> T-cell subset. **a**, Migration of purified T cells and the CD4<sup>+</sup>, CD8<sup>+</sup>, CD45RA<sup>+</sup> and CD45RO<sup>+</sup> T-cell subsets in response to DC-CK1 and RANTES is indicated as the number of migrated cells versus the amount of chemokine. Experiments using filters of 5- $\mu$ m and 8- $\mu$ m pore-size yielded similar results. Results shown are representative from experiments using 5- $\mu$ m pore-size filters. Cells on the filter are counted from 5 fields under high power. Each point represents mean ( $\pm$ s.e.m.) number of migrating cells minus the number of cells in the medium control from 3 experiments performed in duplicate. **b**, **c**, Migration of CD45RA<sup>+</sup> and CD45RO<sup>+</sup> T cells in response to DC-CK1, RANTES, MIP-1 $\alpha$  and IL-8, and their sensitivity to 100 ng ml<sup>-1</sup> pertussis toxin. The concentration of chemokine yielding the optimal response (10 ng ml<sup>-1</sup> for all chemokines) is shown. Results are representative from experiments using 8- $\mu$ m pore-size filters. Each point represents mean ( $\pm$ s.e.m.) number of migrating cells minus the number of cells in the medium control from 3 experiments performed in duplicate.





conditions (Fig. 2b). Splenocytes activated with IL-4 and anti-CD40 monoclonal antibodies also do not express DC-CK1 (data not shown). Taken together, these data demonstrate that DC-CK1 is primarily expressed in dendritic cells but not in any of the other leukocytes tested.

To investigate the time required for the expression of DC-CK1 and the dependence on GM-CSF and IL-4, a time-course experiment was performed using either GM-CSF, IL-4 or a combination of both. No DC-CK1 mRNA could be detected in freshly isolated monocytes, or monocytes cultured for 24 h in any cytokine combination (Fig. 2c). However, after 3.5 days of culture, DC-CK1 mRNA is abundantly present in cells cultured with either GM-CSF plus IL-4 or IL-4 alone, but not with GM-CSF alone. After 6 days, DC-CK1 mRNA is still present in abundance in GM-CSF plus IL-4 and IL-4 cultured cells, whereas only a small amount can be discerned with GM-CSF alone. This latter finding might be explained by the survival of contaminating dendritic cells residing in the monocyte fraction. Collectively, these data indicate that high-level expression of DC-CK1 is dependent on the presence of IL-4. The finding that prolonged incubation in the presence of IL-4 (3.5 days) is required for the induction of DC-CK1 mRNA is consistent with IL-4 being a key cytokine in directing the differentiation of monocytes towards the dendritic cell lineage, whereas GM-CSF is merely thought to act as a survival factor<sup>5,6</sup>.

To determine the number of dendritic cells in our preparations that express DC-CK1 mRNA, we did *in situ* hybridization (ISH) on cytopins of dendritic cell cultures. The results demonstrated that most cells (65–90%) expressed DC-CK1 mRNA, excluding the possibility of a contaminating cell population being responsible for its production (data not shown). To investigate the expression of DC-CK1 *in situ*, ISH was performed on secondary lymphoid tissues, which are known to contain dendritic cells. DC-CK1-expressing cells were readily observed in T-cell areas (identified by CD34 staining of high endothelial venules<sup>8</sup>) and germinal centres of a tonsil (Fig. 3), consistent with the distribution and morphology of dendritic cells<sup>1,9</sup>. A similar expression pattern of DC-CK1 mRNA was observed in lymph nodes. Staining of serial sections for DC-CK1 by ISH and with monoclonal antibodies directed against CD68 and DRC-1/CD21 demonstrated that the DC-CK1-producing cells in the germinal centres were distinct from tingible body macrophages and follicular dendritic cells, respectively (data not shown). As determined with anti-CD3, -CD19 and -CD14 monoclonal antibodies, the DC-CK1-positive cells are also distinct from T cells, B cells and monocytes (data not shown). The DC-CK1-expressing dendritic cells in germinal centres are likely to represent the recently identified T-cell stimulatory germinal-centre dendritic cells<sup>9</sup>.

Because dendritic cells are so important for the activation of unprimed T cells, we investigated the ability of DC-CK1 to function as a chemoattractant for different T-cell subsets. Freshly isolated T cells respond optimally to 10 ng ml<sup>-1</sup> DC-CK1 (Fig. 4c), which is similar to the amount required for the potent T-cell chemoattractant RANTES. CD4<sup>+</sup> and CD8<sup>+</sup> T cells respond equally to DC-CK1. In contrast, separation of T lymphocytes into resting (CD45RA<sup>+</sup>) or activated (CD45RO<sup>+</sup>) subpopulations demonstrated that the main T-cell population attracted by DC-CK1 consists of naive resting T cells. Checkerboard analysis demonstrated that the effect of DC-CK1 on resting T cells is chemotactic rather than chemokinetic in nature (data not shown). The T-cell chemoattractants RANTES, MIP-1 $\alpha$  and IL-8 do not show this specificity for naive CD45RA<sup>+</sup> T cells<sup>10–12</sup> (Fig. 4b). DC-CK1 may therefore be used by dendritic cells to preferentially attract naive T cells which, after recognition of peptide/MHC complexes presented by dendritic cells results in the induction of a primary immune response.

Chemokines induce chemotaxis when they interact with specific receptors that signal through heterotrimeric G proteins. As a consequence, chemotaxis is sensitive to pertussis toxin, a potent inhibitor of G $\alpha_i$  proteins<sup>13</sup>. Pretreatment of cells with pertussis toxin

completely abrogated the chemotactic response of CD45RA<sup>+</sup> T cells to DC-CK1 (Fig. 4c). These data provide further evidence that DC-CK1 acts primarily on the CD45RA<sup>+</sup> T-cell subset, and indicate that the response to DC-CK1 is a G protein-coupled receptor-mediated event.

The specific expression of a chemokine, DC-CK1, preferentially attracting naive T cells may be one of the mechanisms used by dendritic cells to interact preferentially with unprimed T cells, and is likely to be an important first step in the initiation of an immune response. Monoclonal antibodies directed against DC-CK1 will be useful in analysing the lineage relationship between subsets of dendritic cells, as well as in defining stages of their activation and maturation<sup>14</sup>. Further, such antibodies will be an important tool for detecting dendritic cells in different pathological conditions, including cancer and HIV infection. Notably, HIV-1 infection has recently been shown to be suppressed by chemokines and to be dependent on chemokine receptors as a cell entry cofactor<sup>15,16</sup>. Further characterization of DC-CK1 and its receptor will make clear its function as an immunoregulator and its importance in the biology of dendritic cells, including their role and that of DC-CK1 in the pathogenesis of HIV.  $\square$

## Methods

**Cell culture.** Dendritic cells were generated by culturing elutriated monocytes<sup>17</sup> (>90% CD14<sup>+</sup>) in Iscove's medium with 5% FCS, 800 U ml<sup>-1</sup> GM-CSF (Schering-Plough, The Netherlands) and 500 U ml<sup>-1</sup> IL-4 (Schering-Plough) as described<sup>7</sup>. Dendritic were collected directly or after activation with LPS (2  $\mu$ g ml<sup>-1</sup>), TNF- $\alpha$  (15 ng ml<sup>-1</sup>) plus 75 LAF u ml<sup>-1</sup> IL-1 $\alpha$  (Hoffman LaRoche, Nutley, NJ) or 40% monocyte supernatant for 4 h or 16 h. A mixture of the 4-h and 16-h portions was used for northern analysis and cDNA library construction. The phenotype and effects of subsequent stimulation were confirmed by FACS analysis<sup>5–7</sup>. For northern analysis, total PBMC and the non-adherent fraction of PBMC were activated with 1  $\mu$ g ml<sup>-1</sup> PHA and 20 U ml<sup>-1</sup> IL-2 (Cetus, Emeryville) (PBMC-act; Non-Adh act) for 3 days. The adherent PBMC fraction was activated with 2  $\mu$ g ml<sup>-1</sup> LPS for 2 days. Elutriated monocytes (>85% CD14<sup>+</sup>) were activated with 2  $\mu$ g ml<sup>-1</sup> LPS. 75 U ml<sup>-1</sup> IFN- $\gamma$  (Boehringer Ingelheim, Alkmaar, The Netherlands) or 5% FCS for 4 days and elutriated T cells (>95% CD3<sup>+</sup>) with GM-CSF (800 U ml<sup>-1</sup>) and IL-4 (500 U ml<sup>-1</sup>) with or without 1  $\mu$ g ml<sup>-1</sup> PHA and 20 U ml<sup>-1</sup> IL-2 for 3 days. T-cells subsets used for chemotaxis were obtained by negative selection with the appropriate cocktails of monoclonal antibodies against CD14 (Leu-M3), CD19 (Leu-12), CD20 (Leu-16), CD56 (Leu-19), CD45RA (Leu-18), CD45RO (Leu-45RO) (all Becton Dickinson, Mountain View, USA), CD67 (IOM-67, Immunotech, Westbrook), glycophorin (10F7MN, ATCC Rockville), CD4 (OKM-1) and CD8 (BL12) from ficoll banded mononuclear cells using sheep anti-mouse M-450 Dynabeads (Dyna, Oslo, Norway). Purity was >94% as determined by FACS analysis.

**cDNA library construction and northern blot analysis.** Total RNA was isolated from the dendritic cell cultures described above using the guanidine thiocyanate/cesium chloride procedure. After isolation of poly(A)<sup>+</sup> RNA (Oligotex, Qiagen), cDNA was synthesized and cloned into pSport1 using the superscript plasmid system (Gibco BRL). Nucleotide sequence analysis of 360 randomly picked clones was performed using the ABI system. For northern analysis, RNA isolated using the RNazol B method (Biotech Lab., Houston, TX) was used. As a DC-CK1-specific probe, we used a random primed labelled 380-bp BSTX-I/*Not*I fragment comprising the 3' non-coding region. Spotblots containing MIP-1 $\alpha$  cDNA were included in each hybridization to ensure specificity. To control for the amount and quality of the RNA samples, blots were also hybridized with a 28S ribosomal probe<sup>18</sup>.

**Recombinant chemokines and chemotaxis.** The N terminus of DC-CK1 was defined by N-terminal sequencing of purified recombinant DC-CK1 produced in COS-7 cells using the pFLAG system (International Biotechnologies, New Haven, CT). To produce recombinant DC-CK1 used in the chemotaxis experiments, *Escherichia coli* strain X156F was transformed with the pOMP plasmid containing the mature DC-CK1 coding region. After lysis, the periplasmic fraction was purified on Q-sepharose and S-sepharose columns (Pharmacia) using a linear NaCl gradient (0–0.1 M). The DC-CK1-enriched



fractions were loaded on a reverse-phase column and eluted using a linear gradient of 2–80% acetonitrile. DC-CK1 protein concentration was estimated by densitometric scanning of a coomassie blue-stained gel containing lysozyme as a standard. RANTES, MIP-1 $\alpha$  and IL-8 were obtained from R&D Systems. T-cell migration was measured using 48-well chemotaxis chambers (Neuroprobe) as described<sup>19</sup>. In brief, chemokines in RPMI-1640 were added to the lower chamber and were separated from 10<sup>5</sup> cells in RPMI-1640 with 10% FCS by either a 8- $\mu$ m or a 5- $\mu$ m PVP-free polycarbonate membrane (Poretics, Livermore). After incubation for 1 h, the membrane was removed and the upper side washed with PBS, scraped to remove residual cells and washed again. After methanol fixation and staining, the number of fully migrated cells was counted microscopically in 5 high-power fields ( $\times 400$ ) per well. Pertussis toxin (100 ng ml<sup>-1</sup>) (Calbiochem) was used for 2 h. Each experiment was performed in duplicate, and experiments with DC-CK1, RANTES, MIP-1 $\alpha$  and IL-8 were performed in parallel in the same assay to make a direct comparison of their activities possible.

**In situ hybridization.** Cryosections (8  $\mu$ m) of tonsils and lymph nodes were fixed in 4% paraformaldehyde and pretreated with 2  $\mu$ g ml<sup>-1</sup> pepsin in 0.2 M HCl for 10 min and 0.1 M triethanol amine/0.25 acetic acid anhydride for 10 min. Sections were hybridized overnight with either a sense or an antisense DIG-labelled DC-CK1 RNA probe consisting of the 3' non-coding region generated by *in vitro* transcription (Boehringer Mannheim). Before incubation with anti-DIG-alkaline phosphatase monoclonal antibody the sections were treated with 40 U ml<sup>-1</sup> RNase I (Promega) to ensure specificity. After incubation for 2–3 h with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) the sections were stained with methylene green and embedded in Kaiser's. Immunostaining was performed as described<sup>20</sup>.

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Correspondence and requests for materials should be addressed to G.J.A. (e-mail: g.adema@dent.kun.nl).

## A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor

Robert D. Klein<sup>\*†</sup>, Daniel Sherman<sup>\*</sup>, Wei-Hsien Ho<sup>\*</sup>, Donna Stone<sup>\*</sup>, Gregory L. Bennett<sup>†</sup>, Barbara Moffat<sup>‡</sup>, Richard Vandlen<sup>‡</sup>, Laura Simmons<sup>§</sup>, Qimin Gu<sup>§</sup>, Jo-Anne Hong<sup>||</sup>, Brigitte Devaux<sup>||</sup>, Kris Poulsen<sup>\*</sup>, Mark Armanini<sup>\*</sup>, Chika Nozaki<sup>§</sup>, Naoya Asai<sup>§</sup>, Audrey Goddard<sup>§</sup>, Heidi Phillips<sup>\*</sup>, Chris E. Henderson<sup>#</sup>, Masahide Takahashi<sup>§</sup> & Arnon Rosenthal<sup>\*</sup>

Departments of <sup>\*</sup>Neuroscience, <sup>†</sup>BioAnalytical Technology, <sup>‡</sup>Protein Biochemistry, <sup>§</sup>Molecular Biology and <sup>||</sup>Antibody Technology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA  
<sup>§</sup>Department of Pathology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan  
<sup>#</sup>INSERM U.382, Developmental Biology Institute of Marseille, 13288 Marseille 09, France  
<sup>§</sup>Present address: Deltagen, Inc., 1031 Bing Street, San Carlos, California 94070, USA

Glial-cell-line-derived neurotrophic factor (GDNF) and neurturin (NTN) are two structurally related, potent survival factors for sympathetic, sensory and central nervous system neurons<sup>1–6</sup>. GDNF mediates its actions through a multicomponent receptor system composed of a ligand-binding glycosyl-phosphatidylinositol (GPI)-linked protein (designated GDNFR- $\alpha$ ) and the transmembrane protein tyrosine kinase Ret<sup>7–12</sup>. In contrast, the mechanism by which the NTN signal is transmitted is not well understood. Here we describe the identification and tissue distribution of a GPI-linked protein (designated NTNR- $\alpha$ ) that is structurally related to GDNFR- $\alpha$ . We further demonstrate that NTNR- $\alpha$  binds NTN ( $K_d \sim 10$  pM) but not GDNF with high affinity; that GDNFR- $\alpha$  binds to GDNF but not NTN with high affinity; and that cellular responses to NTN require the presence of NTNR- $\alpha$ . Finally, we show that NTN, in the presence of NTNR- $\alpha$ , induces tyrosine-phosphorylation of Ret, and that NTN, NTNR- $\alpha$  and Ret form a physical complex on the cell surface. These findings identify Ret and NTNR- $\alpha$  as signalling and ligand-binding components, respectively, of a receptor for NTN and define a novel family of receptors for neurotrophic and differentiation factors composed of a shared transmembrane protein tyrosine kinase and a ligand-specific GPI-linked protein.

In searching for a neurturin receptor, we examined sequences deposited in public databases for similarity to the GDNF receptor  $\alpha$  component (GDNFR- $\alpha$ )<sup>9,10</sup>. Eight partial human cDNAs (Genbank accession numbers R02249, H12981, W73681, W73633, H05619, R02135, T03342 and HSC1KA111) were identified and found to encode parts of a single protein of 464 amino acids which we designated neurturin receptor  $\alpha$  (NTNR- $\alpha$ ). The human proteins hNTNR- $\alpha$  and hGDNFR- $\alpha$  display an overall 48% similarity, and the positions of their cysteine residues are conserved (Fig. 1). Both hNTNR- $\alpha$  and hGDNFR- $\alpha$  seem to be extracellular proteins that are attached to the outer cell membrane by means of a glycosyl-phosphatidyl inositol (GPI) modification. hNTNR- $\alpha$  has an amino-terminal signal peptide for secretion, three glycosylation sites, and a stretch of 17 carboxy-terminal hydrophobic amino acids preceded by a group of three small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (Fig. 1). Subsequently, a rat NTNR- $\alpha$  (rNTNR- $\alpha$ ) was isolated and shown to be 94% identical to its human homologue (Fig. 1).

To examine whether NTNR- $\alpha$  could be a receptor for a neurotrophic factor such as NTN, the tissue distribution of NTNR- $\alpha$  mRNA was examined by *in situ* hybridization and compared with